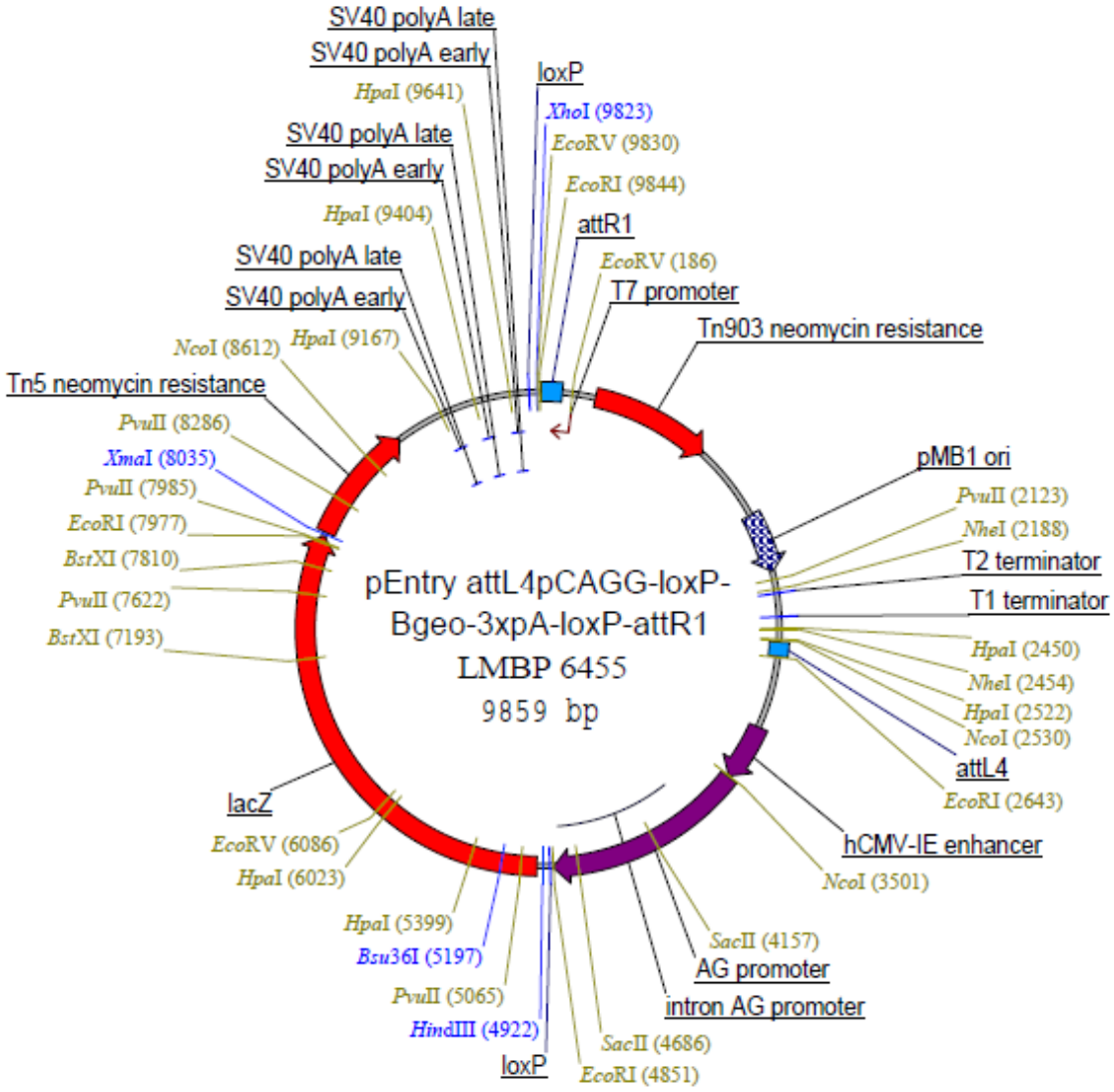
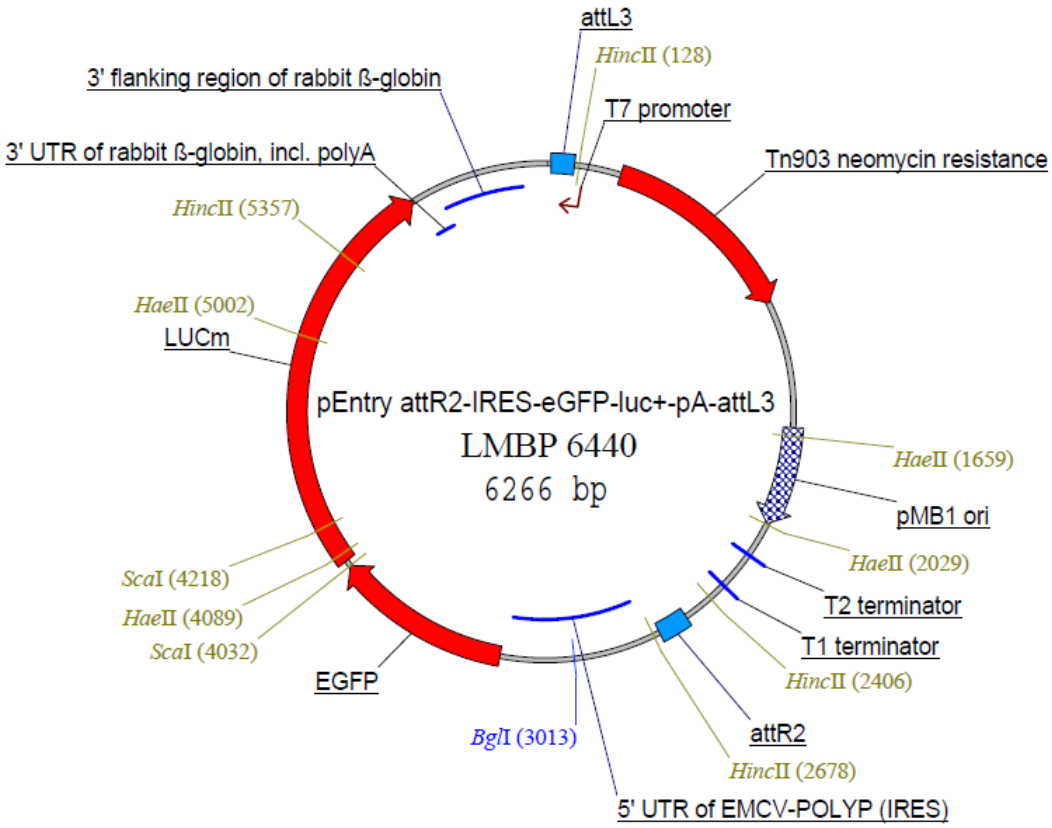
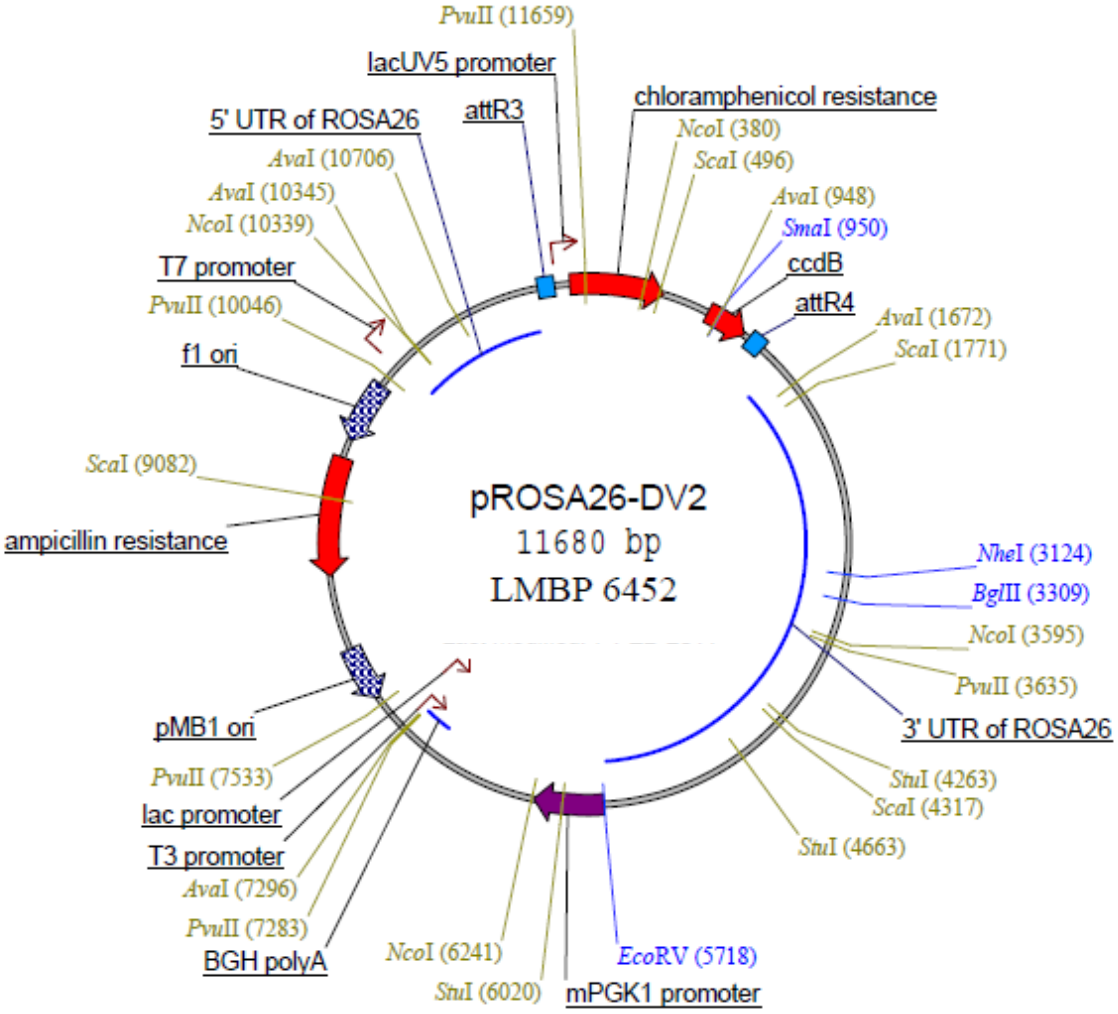
Appendix

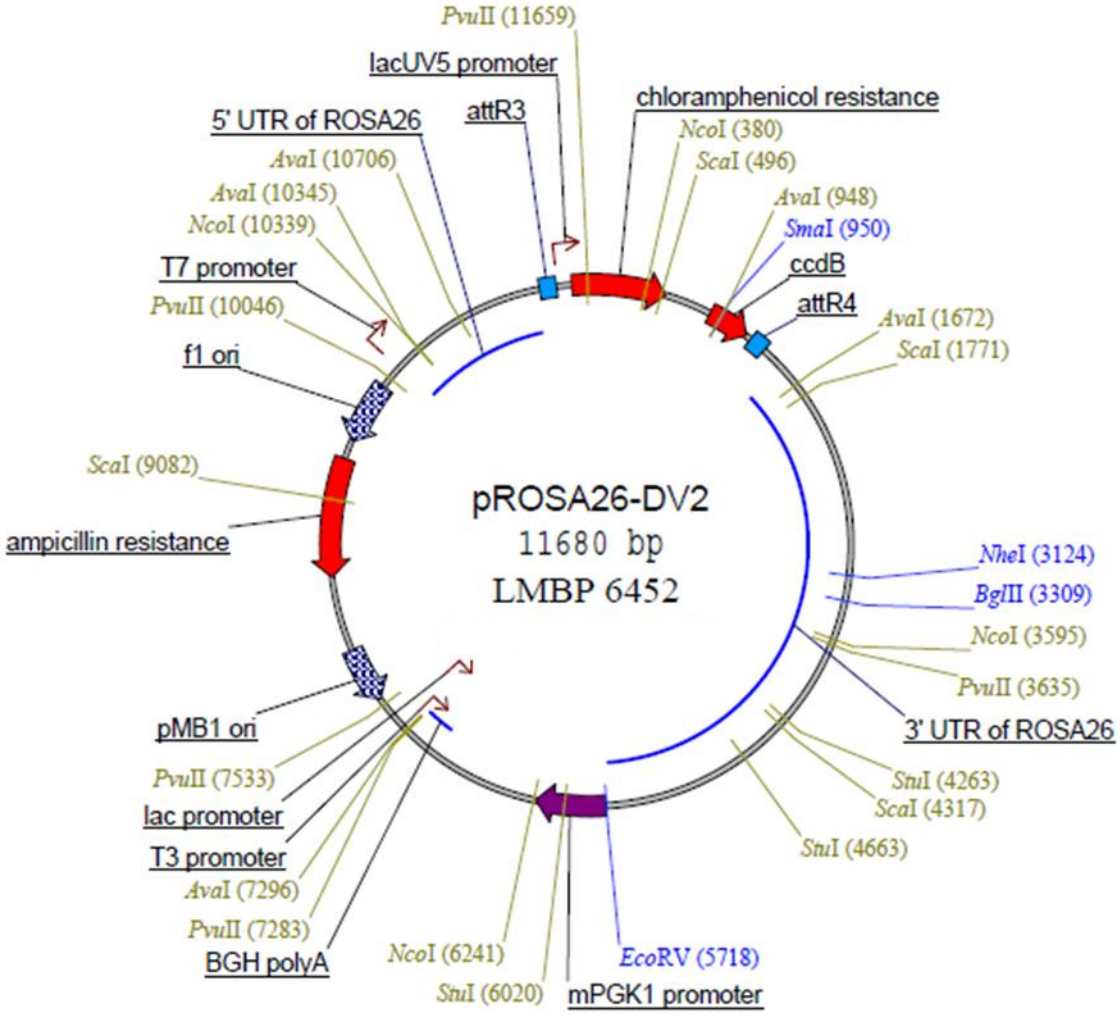
Appendix 1: Plasmid Maps

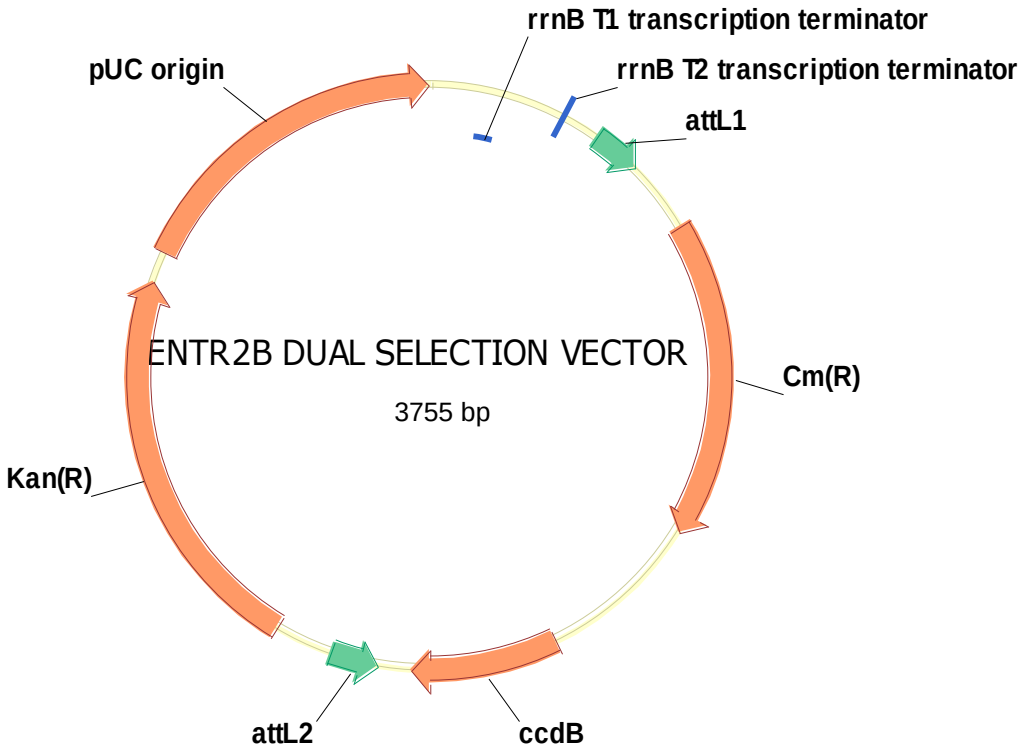
1.1 Plasmids for targeting

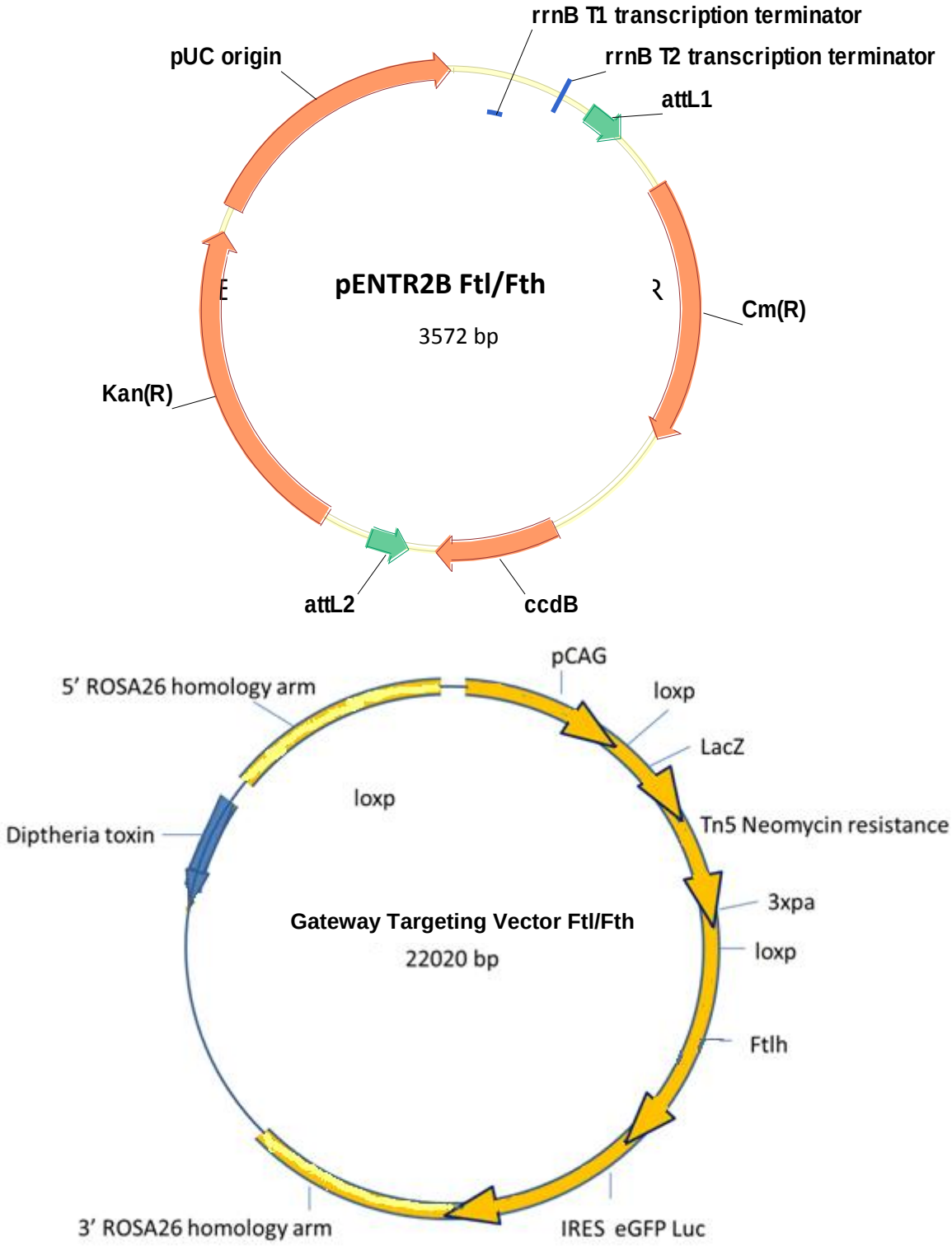



attL3
HincII (128)
T7 promoter
Tn903 neomycin resistance
3' flanking region of rabbit ß-globin
3' UTR of rabbit ß-globin, incl. polyA
HincII (5357)
HaeII (5002)
LUCm
pEntry attR2-IRES-eGFP-luc+-pA-attL3
LMBP 6440
6266 bp
HaeII (1659)
pMB1 ori
HaeII (2029)
T2 terminator
T1 terminator
HincII (2406)
attR2
HincII (2678)
5' UTR of EMCV-POLYP (IRES)
BglI (3013)
EGFP
ScaI (4218)
HaeII (4089)
ScaI (4032)

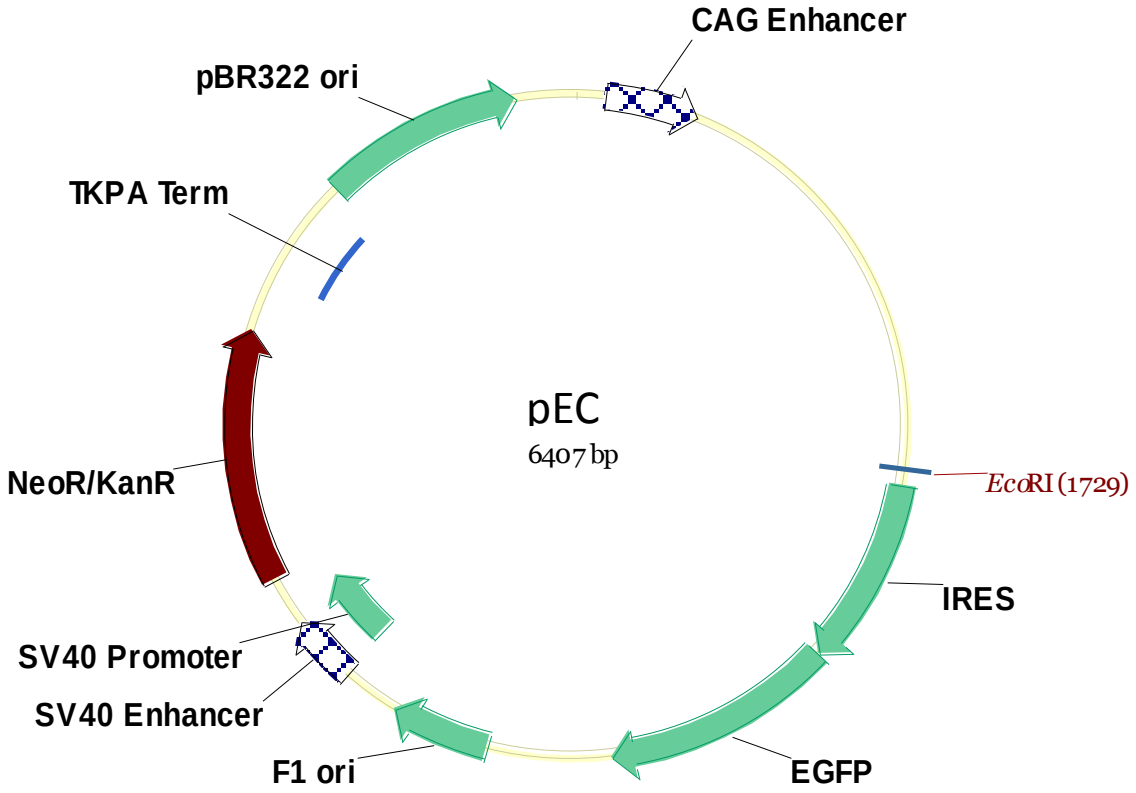

pROSA26-DV2
11680 bp
LMBP 6452
PvuII (11659)
lacUV5 promoter
attR3
5' UTR of ROSA26
AvaI (10706)
AvaI (10345)
NcoI (10339)
T7 promoter
PvuII (10046)
f1 ori
ScaI (9082)
ampicillin resistance
pMB1 ori
PvuII (7533)
lac promoter
T3 promoter
AvaI (7296)
PvuII (7283)
BGH polyA
NcoI (6241)
StuI (6020)
EcoRV (5718)
mPGK1 promoter
chloramphenicol resistance
NcoI (380)
ScaI (496)
AvaI (948)
SmaI (950)
ccdB
attR4
AvaI (1672)
ScaI (1771)
NheI (3124)
BglII (3309)
NcoI (3595)
PvuII (3635)
3' UTR of ROSA26
StuI (4263)
ScaI (4317)
StuI (4663)

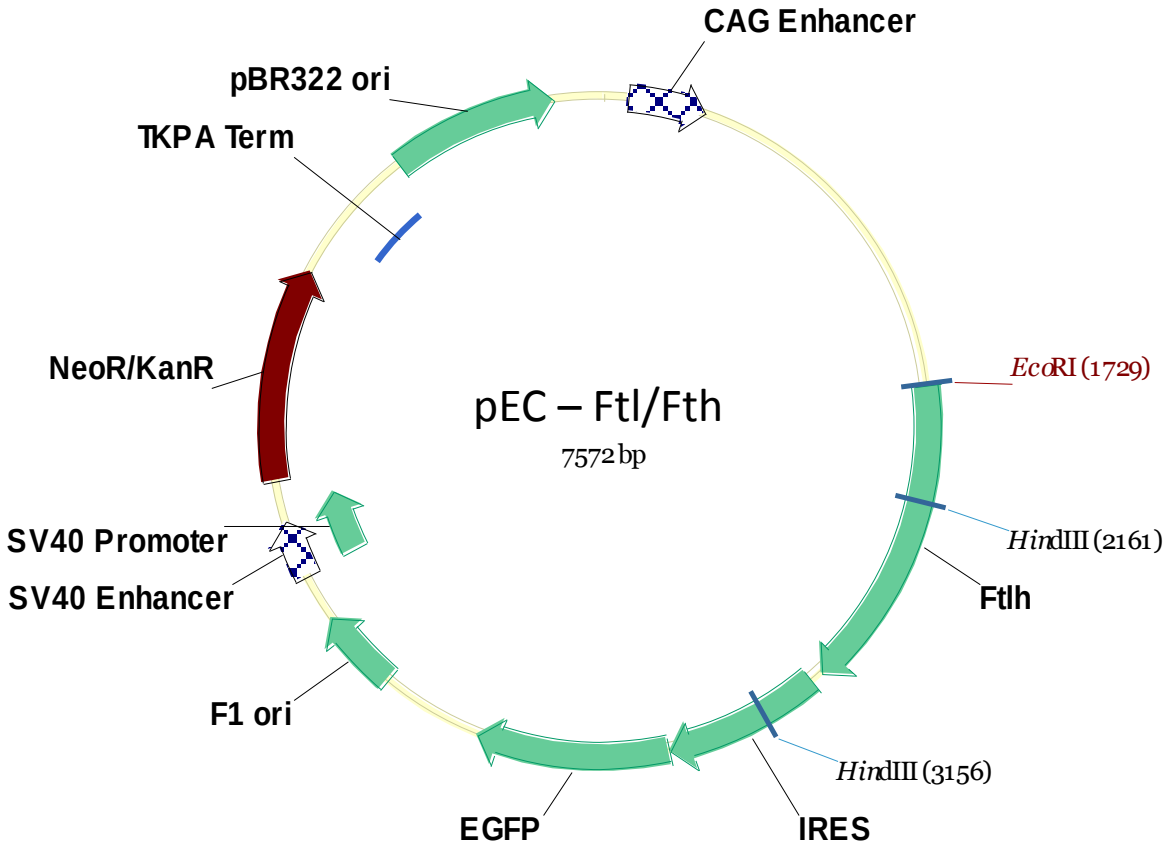

PvuII (11659)
lacUV5 promoter
attR3
chloramphenicol resistance
5' UTR of ROSA26
NcoI (380)
AvaI (10706)
ScaI (496)
AvaI (10345)
AvaI (948)
NcoI (10339)
SmaI (950)
T7 promoter
ccdB
PvuII (10046)
attR4
AvaI (1672)
f1 ori
ScaI (1771)
ScaI (9082)
pROSA26-DV2
11680 bp
LMBP 6452
ampicillin resistance
NheI (3124)
BglII (3309)
NcoI (3595)
PvuII (3635)
3' UTR of ROSA26
pMB1 ori
PvuII (7533)
StuI (4263)
lac promoter
ScaI (4317)
T3 promoter
StuI (4663)
AvaI (7296)
PvuII (7283)
NcoI (6241)
EcoRV (5718)
BGH polyA
StuI (6020)
mPGK1 promoter


rrnB T1 transcription terminator
pUC origin
rrnB T2 transcription terminator
attL1
ENTR2B DUAL SELECTION VECTOR
3755 bp
Cm(R)
Kan(R)
attL2
ccdB

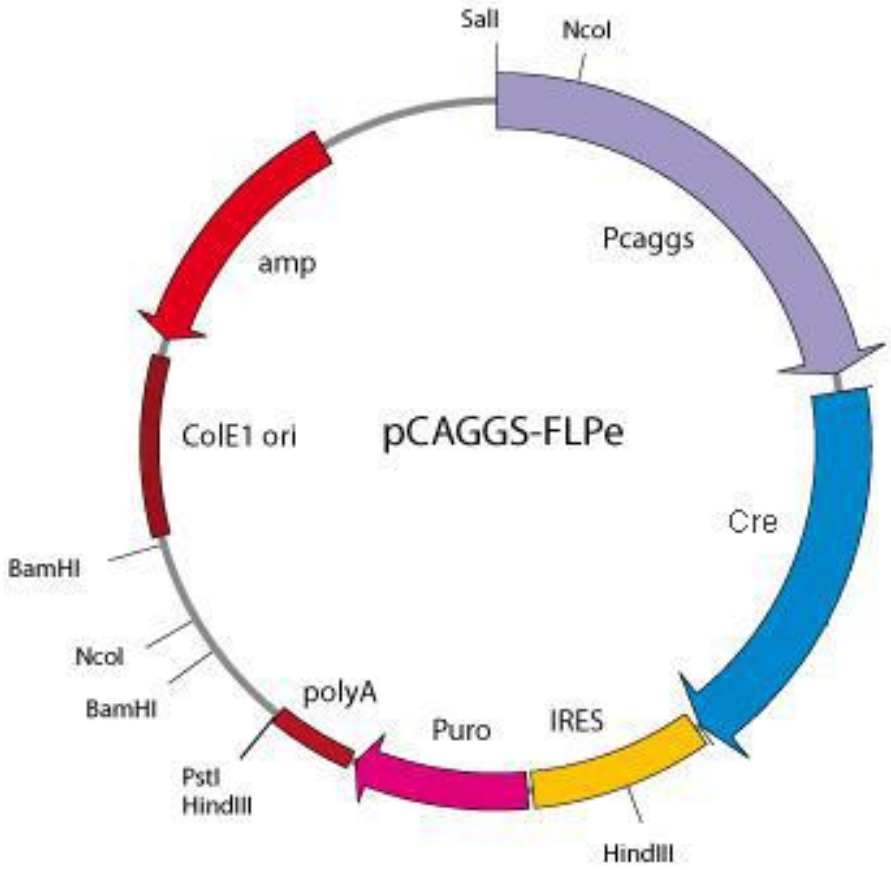

rrnB T1 transcription terminator
rrnB T2 transcription terminator
pUC origin
attL1
pENTR2B Ftl/Fth
3572 bp
Cm(R)
Kan(R)
attL2
ccdB
pCAG
5' ROSA26 homology arm
loxp
LacZ
loxp
Diptheria toxin
Tn5 Neomycin resistance
3xpa
Gateway Targeting Vector Ftl/Fth
22020 bp
loxp
Ftlh
3' ROSA26 homology arm
IRES eGFP Luc

1.2 Plasmids for random integration




CAG Enhancer
pBR322 ori
TKPA Term
NeoR/KanR
pEC – Ftl/Fth
7572 bp
EcoRI (1729)
HindIII (2161)
Ftlh
HindIII (3156)
IRES
EGFP
F1 ori
SV40 Promoter
SV40 Enhancer

1.3 Cre Plasmid



Appendix 2: Preparation of the LIF plasmid

The LIF expression vector (Ampicilin resistant) was previously donated by Vladamir Buchman. It was transformed into TOP 10 competent cells using heat shock at 42°C for 1 min, then transfer to ice for 2 min and purified by Endofree Maxi prep (Qiagen) for further details see Chapter 2. The LIF was transfected using lipofection into COS cells. COS cells were maintained in DMEM supplemented with 10% foetal calf serum, 1% Pen/Strep and L-Glutamine with media changes every two to three days and passaging at 80% confluence using Trypsin-EDTA. At 60-70% confluence cells were transfectred and a few hours prior to transfection, media was changed for that containing no Pen/Strep.

Using one 6cm dish 8µg of DNA was added into 50µl Opti-MEM. Lipofectamine was mixed before use. 20µl Lipofectamine was added into 50µl Opti-MEM and incubated for 5mins at room temperature. The DNA containing Opti-MEM was then combined with the Lipofectamine, mixed gently and incubated at room temperature for 20mins (solution may appear cloudy). This was added to the cells with 2 mls of OptiMEM and incubated at 37°C for 24 hours.

After 24 hours the transfection mix was discarded and 10ml ES cell medium without LIF or Pen/Strep was added and left over night at 37°C. The media was collected into 50ml tube the next day (kept at 4°C). A further 10ml ES cell medium without LIF or Pen/Strep was added to the cells and left at 37°C overnight. Once again this media was collected and pooled with the previous batch of media and filter sterilised. The media was stored in aliquots at -20°C.

The LIF conditioned media was tested at various concentrations in clonally plated mouse ES cell cultures, in 6-well plates and compared against 'No LIF' and 'old LIF', to determine the optimal concentration of 'new LIF' to use in ES cell medium. Leishman's stain was used to determine the optimal concentration of LIF.

Appendix 3: Recipes

Molecular Antibiotic Concentrations

Ampicilin: 100 µg/ml and for LIF production 150µg/ml

Kanamycin: 30-50 µg/ml

Chloramphenicol: 15-30 µg/ml

Tissue Culture Antibiotic Concentrations

G418 (Geneticin) 200µg/ml (Sigma)

Puromycin 1ng/ml (Sigma)

Southern Blot Specific Solutions

20xSSC

175.3g NaCl
88.2g Na citrate
800ml H20
pH 7.0
Adjust to 1 litre with H_20
Autoclave sterilize

Western Blot Specific Solutions

Lysis Buffer

1ml RIPA Buffer (Sigma), Complete Mini 1x solution (Roche)

Protein assay.

Want 20-40ug/lane (max. vol 30µl)

Western blotting buffers

10x running buffer

0.25M Tris base, 1.92M glycine, 0.1% SDS pH 8.3

1x TBS (Tris buffered saline)

10mM Tris, 150mM NaCl, pH 7.6

Transfer buffer

0.25M Tris base, 1.92M glycine, 20% methanol

Sample loading buffer

2% SDS, 10% glycerol, 60mM Tris base pH 6.8, 0.005% Bromophenol blue, 500mM DTT

Resolving gel

12% Acrylamide (Sigma-Aldrich), 0.37M Tris, 0.1% SDS, 0.1% Ammonium persulphate (APS), 0.06% tetramethylethylenediamine (TEMED) (Sigma- Aldrich).

Stacking Gel

5% Acrylamide (Sigma-Aldrich), 0.125M Tris, 0.1% SDS, 0.05% APS, 0.5% TEMED (Sigma- Aldrich).

Ponceau S

0.1% in 5% acetic acid

Wash buffer

1x TBS, 0.1% Tween 20 (Sigma)

Antibody blocking solution

1% skimmed milk powder (Marvel, UK) in 1x TBS, 0.1% Tween 20 (Sigma)

Ponceau S

0.1% in 5% acetic acid

Antibody blocking solution

1% marvel in TBS/Tween + 100μl sodium azide (1M) per 20ml

Rainbow marker (GE Healthcare)

10μl / lane

Gel running

Either 200V for 30min (100V for 1h) or 100V stacking gel (15min) 150V resolving gel (1h)

Quinolinic Acid

QA was dissolved in 0.1M phosphate buffer to make a 90mM solution. The working concentration was 45nmol.

6-Hydroxydopamine

One gram of 6-OHDA (hydrobromide salt) (Sigma), was dissolved in 0.8ml of sterile saline, giving a 30mM solution of 6-OHDA with 0.03% ascorbic acid as a stabilizer and a concentration of 5.14 mg/ml of the free base. The dissolved neurotoxin was frozen as 100µl aliquots for future use and when required they were thawed quickly and stored on ice during surgery for a maximum of two hours after which they were discarded.

Fixative (4% paraformaldehyde)

40g PFA
1L Prewash Buffer
Heat with addition of NaOH to dissolve
pH 7.3